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Design and synthesis of 7-alkoxy-4-heteroarylamino-3-quinolinecarbonitriles as dual inhibitors of *c*-Src kinase and nitric oxide synthase

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Abstract—Because both *c*-Src and iNOS are key regulatory enzymes in tumorigenesis, a new series of 4-heteroarylamino-3-quinolinecarbonitriles as potent dual inhibitors of both enzymes were designed, prepared, and evaluated for blocking multiple signaling pathways in cancer therapy. All compounds were evaluated by two related enzyme inhibition assays and an anti-proliferation assay in vitro. The results showed that most compounds could inhibit both enzymes, and several of them showed potent inhibition activity against different cancer cell lines. The best compound **20** (CPU-Y020) showed the IC_{50} values of 6.58 and 7.61 μM toward colon cancer HT-29 and liver cancer HepG2 cell lines.
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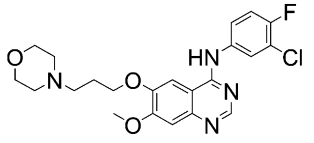
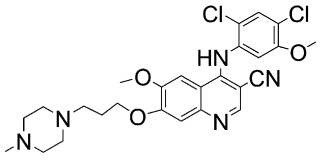
1. Introduction

The Src family of protein tyrosine kinases (SFKs) plays key roles in regulating signal transduction, including cell growth, differentiation, cell shape, migration, and survival, and specialized cell signals.[1] However, *c*-Src was also identified as a proto-oncogene based on decades of research on an avian RNA tumor (sarcoma) virus. In some abnormal cases, such as mutation of the *c*-Src or over-expression, these enzymes can become hyperactivated, resulting in uncontrolled cell proliferation.[2]

Inhibition of Src kinase activity to block uncontrolled cell growth has potential therapeutic implications in developing cancer treatments. This research has achieved major progress in this regard, and several quinoline/quinazoline compounds were identified as potent and selective inhibitors of PTKs. For example, gefitinib (Iressa, ZD-1839, Astra Zeneca) was launched in 2003, and bosutinib (SKI-606, Wyeth), a dual inhibitor of Src and Abl kinases based on a 3-quinolinecarbonitrile skeleton, is currently in phase II clinical trials (Fig. 1).[3–5]



Iressa (ZD-1839)



Bosutinib (SKI-606)

Figure 1. The structures of Iressa (ZD-1839) and Bosutinib (SKI-606).

iNOS is an inducible isoform of nitric oxide synthase (NOS), expressed in a wide range of cells and tissues such as macrophages,[6] Kupffer cells, colonic epithelium, vasculature,[7] and various neoplastic tissues.[8] iNOS is regulated by a variety of factors including bacterial lipopolysaccharides (LPS), cytokines, and signal trans-

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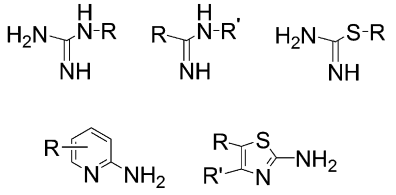
duction enzymes. iNOS highly expresses in various neoplastic diseases. The role of iNOS during tumor development is highly complex, and incompletely understood. But there is no doubt that the malignant transformation, angiogenesis, and metastasis effects of tumors are all modulated by iNOS.[7]

To avoid complications resulting from inhibition of normal physiological NO production and to dissect the natural pharmacological effects, compounds are needed that selectively inhibit iNOS. Fortunately, many NOS inhibitors, such as 2-aminothiazole and 2-aminopyridine derivatives (Fig. 2), have been reported[9,10] to exhibit potent inhibition with variable selectivity for iNOS and its isoforms.

The attempt to suppress two distinct cancer-related enzymes with a single agent was an attractive idea. It is reasonable that the dual inhibitors of both Src and iNOS were more effective than single inhibitors as blocking multiple signaling pathways in cancer therapy and could be beneficial to overcome drug resistance. With the goal of identifying such a new anti-cancer strategy, a new class of dual inhibitors of both enzymes were designed (Fig. 3). Using the potent Src inhibitor template 4-aniline-3-quinolinecarbonitrile as the leading skeleton, the existing 4-anilines were replaced with specialized side groups, such as 2-aminothiazole or 2-aminopyridine derivatives, that have selective inhibition against iNOS. As a result, a new series of 7-alkoxy-4-heteroarylamino-3-quinolinecarbonitriles was designed.



R, R' = alkyl groups, etc.

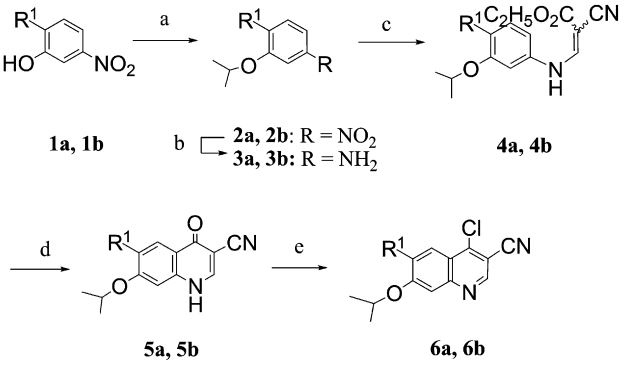
Figure 2. Some selective iNOS inhibitors.

2. Chemistry

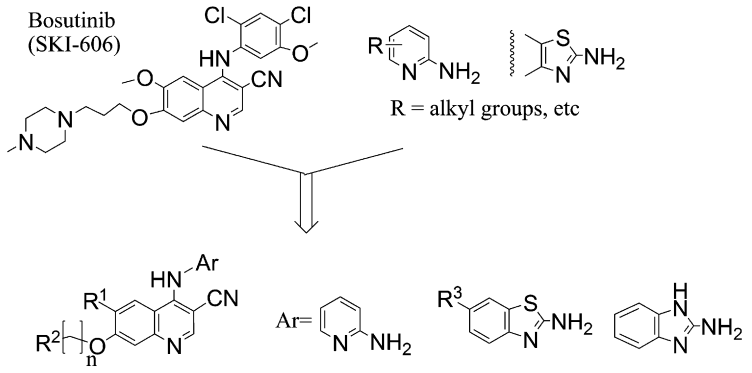
The synthetic pathways used to prepare this series of 7-alkoxy-4-heteroarylamino-3-quinolinecarbonitrile compounds are shown in Schemes 1 and 2. The hydroxyl groups of 2-methyl-5-nitrophenol (**1a**) and 5-nitroguaiacol (**1b**) needed to be protected during the quinoline synthesis, and isopropyl was chosen as the protective group for its good thermal stability in a high temperature quinolones cycling reaction and its convenient deprotection method.

Following the designed methods (Scheme 1),[11,12] the 3-cyano-2-hydro-quinolin-4 (1H)-ones (**5a**, **5b**) were readily made. The core quinolones were aromatized in refluxing $POCl_3$ and the key intermediate 3-cyano-4-chloro-quinolines (**6a**, **6b**) were produced.

The different 4-heteroarylaminos were brought into the skeleton using NaH in anhydrous DMF. Then the 7-isopropyl of the intermediates was deprotected by HBr in an acetic acid solution, yielding the 4-heteroarylamino-7-hydroxyquinolines (**10**, **11a–d**, and **12a–c**) (Scheme 2). It was reported[4] that a morpholinoalkoxy group

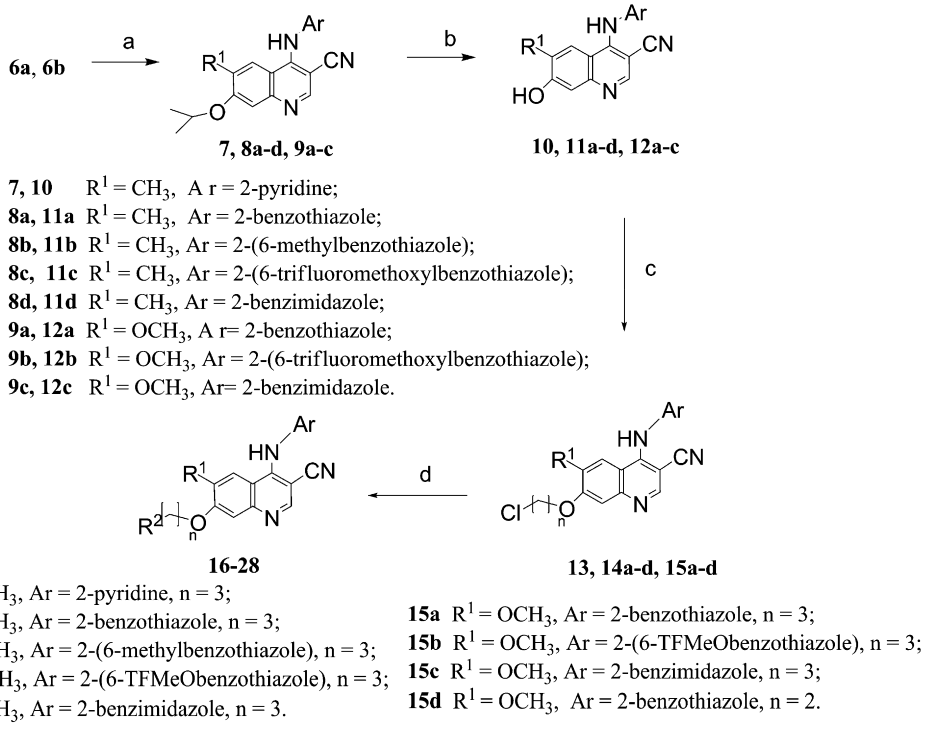


Scheme 1. **1a–6a**: R^1 = Me, **1b–6b**: R^1 = OMe. Reagents and conditions: (a) 2-bromopropane, NaOH, DMF; (b) Fe, HOAc, MeOH; (c) toluene, reflux; (d) liquid paraffin, 260 °C; (e) $POCl_3$, reflux.



n = 2, 3; R^1 = CH_3O, CH_3; R^2 = piperidine, piperazine, etc;
R^3 = H, CH_3, OCF_3.

Figure 3. The design of the dual inhibitors of Src and iNOS.



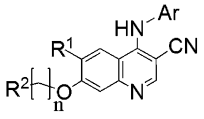
Scheme 2. Reagents and conditions: (a) heteroaromatic amines, NaH, anhydrous DMF; (b) HBr, HOAc, 120 °C; (c) 1-bromo-3-chloropropane or 1-bromo-2-chloroethane, K_2CO_3, DMF; (d) secondary amine (piperazine, piperidine, or morpholine, etc), K_2CO_3, DMF.

substituted on the 7-position of 4-anilinoquinolines can greatly increase potency against Src kinase; therefore we incorporated morpholinoalkoxy and its bioisosteric groups at the 7-position and the target molecules were achieved (Table 1).

3. Results and discussion

Compounds **16** to **28** were synthesized smoothly, and their water solubilities were improved prominently compared with their intermediates. In order to evaluate the

Table 1. The structures of compounds **16–28** and their enzyme inhibition activities against *c*-Src and iNOS[a]



No.	R^1	*n*	R^2	Ar	*c*-Src, IC_{50} (nM)	iNOS, IC_{50} (μM)[a,b]
16	Me	3	*N*-Me-piperazine	2-Pyridine	75.9	18.6
17	Me	3	Morpholine	2-Benzothiazole	172	139
18	Me	3	Piperidine	2-Benzothiazole	209	412
19	Me	3	*N*-Me-piperazine	2-(6-Me-benzothiazole)	646	12.7
20	Me	3	Piperidine	2-(6-Me-benzothiazole)	15.4	313
21	Me	3	*N*-Me-piperazine	2-(6-TFMeO-benzothiazole)	111	16.3
22	Me	3	*N*-Me-piperazine	2-Benzimidazole	>1000	15.5
23	OMe	3	*N*-Me-piperazine	2-Benzothiazole	186	98
24	OMe	3	*N*-Me-piperazine	2-(6-TFMeO-benzothiazole)	178	65.6
25	OMe	2	Piperidine	2-Benzothiazole	371	44.8
26	OMe	2	Morpholine	2-Benzothiazole	>1000	26.7
27	OMe	2	*N*-Me-piperazine	2-Benzothiazole	9.23	2.18
28	OMe	3	*N*-Me-piperazine	2-Benzimidazole	133	18.1
Bosutinib					1.2	—
L-Canavanine					—	60
2-Aminothiazole					—	18

[a] The two enzyme inhibition assay procedures are listed in the Supplementary Material. The IC_{50} values were means of triplet experiments, and the variabilities were within 10%.

[b] iNOS of mouse macrophage ANA-1.

Src kinase inhibition activities, bosutinib was also synthesized as the reference molecule according to the reported method. Besides, the commercially available compounds *L*-canavanine and 2-aminothiazole were chosen as the positive iNOS inhibitors. Then the target molecules described above were tested in two related enzyme assays: a PTK inhibition assay and an iNOS inhibition assay (see Supplementary Material). The IC_{50} values are shown in Table 1.

The data in Table 1 clearly showed that most compounds exhibited moderate inhibition activities toward both Src and iNOS. In general, the 4-heteroarylamino groups substituted compounds including 4-(benzothiazole-2-amine), 4-(6-methylbenzothiazole-2-amine), and 4-(pyridine-2-amine) as the 4-anilino headpieces showed to be active for Src kinase, though their IC_{50} values against Src were weaker than bosutinib. For iNOS, the inhibition activities of the target molecules were relatively weak compared with the inhibition activities against Src kinase, but some of the compounds were more potent than the inhibition activities of *L*-canavanine and 2-aminothaizole. Several compounds inhibited both enzymes well. Compound **27** showed the best inhibition activities with the IC_{50} values of 9.23 nM and 2.18 μM toward Src and iNOS, respectively. Some of the compounds exhibited good activity against only one enzyme. For example, compounds **16**, **19**, and **21** inhibited iNOS well with the IC_{50} values of 18.6, 12.7, and 16.3 μM, but their Src inhibition IC_{50} values were only 75.9, 646, and 111 nM, which were much lower than that of **27**. The opposite situation was found in compound **20** with the IC_{50} values of 15.4 nM and 313 μM against Src and iNOS, respectively.

With respect to different substituted groups, including R^1, R^2, *n*, and several 4-heteroarylaminos, the last one played the most important role in the different IC_{50} trends against the two enzymes. As shown in Table 1, when the C-6 position was a methoxyl group, n was 3 and the alkaline group was *N*-methylpiperazine, all their substituents except the 4-heteroarylamino headpieces of compounds **23**, **24**, and **28** were the same with bosutinib. Their Src inhibition activities decreased much compared with the reference compound, and all their IC_{50} values were less potent than 100 nM. As for the inhibition of iNOS, compound **23** and **24**'s activities were also not predominant with the IC_{50} values of 98 and 65.6 μM, respectively, and were weaker than the selective iNOS inhibitor *L*-canavanine (IC_{50} = 60 μM). Compound **28** with a 4-(benzimidazole-2-amine) headpiece showed better inhibition activity against iNOS with an IC_{50} of 18.1 μM, and the inhibition activity was similar to 2-aminothiazole (IC_{50} = 18 μM).

Different 4-heteroarylamino groups were also tested with 6-methyl substituted compounds. When n was 3 and the alkaline group was *N*-methylpiperazine, compounds **16**, **19**, **21**, and **22** showed various activities toward the target enzymes. Compound **16** with a pyridine-2-amine substituent at the C-4 position exhibited moderate inhibition activity toward both enzymes with the IC_{50} values of 75.9 nM and 18.6 μM. Compound **21**'s activity against Src improved to 111 nM compared with that of the corresponding 6-methoxy compound **24,** while compound **19**'s activity was about 5-times weaker than that of **21**. When there was a benzimidazole-2-amine at C-4 position, the Src inhibition activity of compound **22** was lost. This may imply that the benzimidazole-2-amine was not suitable for C-4 position. In the meanwhile, their inhibition activities against iNOS kept relatively stabilized between 10 and 20 μM. Interestingly, compound **19** showed the best activity with an IC_{50} value of 12.7 μM and was 1.5-times more potent than that of 2-amniothiazole.

Further investigation of the C-7 alkaline side chains was carried out with 4-(benzothiazole-2-amine) substituted compound **23**. When n changed from 3 to 2, the new compound **27**'s inhibition activity against Src increased about 20-times (IC_{50} = 9.23 nM), and the inhibition activity against iNOS also increased about 40-times (IC_{50} = 2.18 μM) compared with compound **23**. But unfortunately, when R^2 of **27** was changed to piperidine or morpholine, the new compounds **25** or **26**'s inhibition activities against both enzymes decreased a lot. Their Src inhibition activities were even weaker than that of **23**. The iNOS inhibition activities of **25** and **26** were those between *L*-canavanine and 2-aminothiazole.

The changes of 7-alkaline groups were also carried out with their 6-methyl analogs. [Both the piperidine and morpholine substituted 4-(benzothiazole-2-amine) compounds **17** and **18** showed weak inhibition activities toward Src and iNOS.] But when changed the *N*-methylpiperazine of **19** to piperidine, the new compound **20** showed more potent activity against Src kinase with an IC_{50} value of 15.4 nM while its iNOS inhibition activity deceased a lot.

In conclusion, some of the 4-heteroarylamino groups showed to be active for Src kinase, especially compounds **20** and **27** with the 4-(benzothiazole-2-amine) and 4-(6-methylbenzothiazole-2-amine) groups showed remarkable IC_{50} against Src kinase. This may suggest that the hydrophobic pocket at the kinase binding site may accommodate larger groups than the traditional single ring arylamines. Although ATP did not directly interact with the residues around this pocket, it was important for inhibitors to bind compactly at the ATP binding site.

For iNOS, the inhibition by the target molecules was relatively weak. Guanidine moiety was a very crucial pharmacophore for iNOS inhibition. Though 2-aminobenzimidazole or 2-aminobenzthiazole derivatives included the guanidine moiety or its bioisosteres, the 2-amino group was linked to the quinoline C-4 position directly and the other N-1, NH-3, or S-1, NH-3 atoms were fixed by the constrained rings. The guanidine pharmacophore in the designed compounds could not interact with iNOS as the free ones. This may be the reason for the weak inhibition of iNOS.

To further investigate the anti-cancer activities of the target molecules, an anti-proliferation SRB (Sulforho-

Table 2. Anti-proliferation effects of compound **20** (CPU-Y020) compared with bosutinib

Assay parameters[a] (μM)	Compound **20**		Bosutinib	
	Liver, HepG2	Colon, HT-29	Liver, HepG2	Colon, HT-29
GI_{50}	4.85	5.59	1.15	1.17.
IC_{50}	7.61	6.58	3.91	3.87[b]
TGI	9.25	>10	7.66	6.50
LC_{50}	>10	>10	>10	>10

[a] The anti-proliferation assay procedure is listed in the Supplementary Material.
[b] The reported IC_{50} value in Ref. 5 is 1.5 μM.

damine B) assay was also carried out against five tumor cell lines (lung cancer A549, stomach cancer AGS, liver cancer HepG2, colon cancer HT-29, and prostate cancer PC-3).[13] According to the anticancer assay results, compound **20** (CPU-Y020) exhibited good inhibition activities toward HepG2 and HT-29 cell lines with the IC_{50} values of 7.61 and 6.58 μM, and was the most potent compound found (Table 2). Compounds **16**, **24**, **27**, and **28** also showed some anti-proliferation activities in the preliminary screening, but their IC_{50} values were all less potent than 10 μM.

Compound **20** exhibited the best anti-proliferation activity against several tumor cell lines. Specifically, it had an IC_{50} value of 6.58 μM against colon cancer HT-29 cell line, and this was similar to the anti-proliferative activity of bosutinib with an IC_{50} value of 3.87 μM.[5] The similar result was also obtained against liver cancer Hep2 cell line with an IC_{50} value of 7.61 μM compared with that of bosutinib's 3.91 μM.

Compound **20** showed good inhibition activity toward Src, with an IC_{50} value of 15.4 nM, but it did not show good inhibition activity toward iNOS with an IC_{50} value of 313 μM. This may imply that the inhibition mechanism of compound **20** was mainly dominated by the inhibition of Src kinase. Other factors besides enzyme inhibitions, such as different degrees of cell penetration and different polarization of the compounds, may also play key roles in retarding the proliferation of tumor cell lines, and this encouraging result might be expected. Further evaluation of compound **20**'s anticancer mechanism was on the way.

4. Conclusions

In this study, a series of new dual inhibitors of Src kinase and iNOS as novel anticancer agents were proposed. The synthesis of 6, 7-disubstituted-4-heteroarylamino-quinoline-3-carbonitriles and their anticancer activities were also described. Parts of these compounds were effective against both enzymes, and they represented the culmination of our earlier efforts in designing such dual inhibitors. One member of this series, compound **20** (CPU-Y020), was the most promising compound and was selected for additional studies to determine its potential in the treatment of cancer.

5. Experimental

5.1. Chemistry

All purchased starting materials and reagents were used without further purification unless noted. ^{1}H NMR spectra were recorded on an ACF-300 Bruker, or an ACF-500 Bruker instrument. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Mass spectra were recorded on a Shimadzu GC–MS 2010 (EI) or a Mariner Mass Spectrum (ESI). HRMS spectra were recorded on a Agilent HPLC–ESI/TOF. FT IR spectra were collected with a Nicolet Impact 410 instrument (KBr pellet). The HPLC spectra were recorded on a Jasco HPLC system. Uncorrected melting points were determined in open capillary tubes on a Mel-TEMP II melting point apparatus. TLCs and preparative thin-layer chromatography were performed on silica gel GF/UV 254, and the chromatograms were performed on silica gel (200–300 mesh) visualized under UV light at 254 and 365 nm. Most of the reactions were carried out under an inert atmosphere of nitrogen. The reported yields were for purified materials but were not optimized.

Compounds **5b** and **6b** were previously reported in a different route.[12] The synthesis of compounds **2a**–**6a** and **2b**–**6b** described in Scheme 1 are listed in the Supplementary Material.

5.1.1. 7-Isopropoxy-6-methyl-4-(pyridin-2-ylamino)-quinoline-3-carbonitrile (7). To a suspension of NaH (60% in mineral oil, 100 mg, 2.5 mmol) in 15 ml of anhydrous DMF, 2-aminopyridine (235 mg, 2.5 mmol) was added. The mixture was heated to reflux for 10 min, then cooled and **6a** (460 mg, 2.0 mmol) was added. The mixture was heated to reflux for 2 h then cooled to room temperature and partitioned between water and ethyl acetate. The organic layer was washed with dilute aqueous ammonium hydroxide, dried over $MgSO_4$, filtered, and concentrated in vacuum. Ethyl acetate and diethyl ether were added to the residue and the solid was collected to provide 245 mg (42% yield) of 7 as a tan solid. Mp 243 °C; EI-MS (*m*/*z*): 290.1 (M^+); ^{1}H NMR (CD_3OD, 500 MH) δ 1.32–1.34 (d, J = 6 Hz, 6H), 2.41 (s, 3H), 4.60 (m, 1H), 7.24 (t, J = 7 Hz, 1H), 7.36 (s, 1H), 7.74 (d, J = 9 Hz, 1H), 7.94 (m, 1H), 8.61 (s, 1H), 9.01 (d, J = 7 Hz, 1H), 9.36 (s, 1H).

5.1.2. 7-Isopropoxy-6-methyl-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (8a). To a suspension of NaH (60% in mineral oil, 100 mg, 2.5 mmol) in 15 ml of anhydrous DMF, 2-aminobenzothiazole (375 mg, 2.5 mmol) was added. The mixture was heated to reflux for 15 min, then cooled and **6a** (520 mg, 2.0 mmol) was added. The mixture was heated at reflux for 3 h then cooled to room temperature and partitioned between water and ethyl acetate. The organic layer was washed with dilute aqueous ammonium hydroxide, dried over $MgSO_4$, filtered and concentrated in vacuum. Ethyl ace-

tate and diethyl ether were added to the residue and the solid was collected to provide 345 mg (46%) of **8a** as a tan solid. Mp 234 °C; EI-MS (*m*/*z*): 374.1 (M^+); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.39–1.41 (d, J = 6 Hz, 6H), 1.41 (s, 3H), 2.37 (s, 3H), 4.90 (m, 1H), 7.41 (s, 1H), 7.53 (m, 2H), 7.99 (d, J = 8 Hz, 1H), 8.42 (s, 1H), 9.48 (d, J = 8 Hz, 1H), 9.59 (s, 1H), 10.00 (s, 1H).

5.1.3. 7-Isopropoxy-6-methyl-4-(6-methylbenzothiazol-2-ylamino)-quinoline-3-carbonitrile (8b). The compound was prepared with a 65% yield according to the method for **8a** using **6a** and 6-methyl-2-aminobenzothiazole. Mp 246 °C; EI-MS (*m*/*z*): 390.1 (M^+); ^{1}H NMR (DMSO-d_6,500 MHz) δ 1.39–1.41 (d, J = 6 Hz, 6H), 2.31 (s, 3H), 2.43 (s, 3H), 4.89 (m, 1H), 7.39 (m, 2H), 7.81 (s, 1H), 8.42 (s, 1H), 9.32 (d, J = 9 Hz, 1H), 9.60 (s, 1H), 9.98 (br s, 1H).

5.1.4. 7-Isopropoxy-6-methyl-4-(6-trifluoromethoxybenzothiazol-2-ylamino)-quinoline-3-carbonitrile (8c). The compound was prepared with a 45% yield according to the method for **8a** using **6a** and 6-trifluoromethoxy-2-aminobenzothiazole. Mp 254–256 °C; EI-MS (*m*/*z*): 458.1 (M^+);^{1}H NMR ($CHCl_3$,300 MHz) δ 1.39–1.42 (d, J = 6 Hz, 6H), 2.37 (s, 3H), 4.88–4.92 (m, 1H), 7.42 (s, 1H), 7.56–7.59 (d, J = 8.4 Hz, 1H), 8.17 (s, 1H), 8.41 (s, 1H), 9.55–9.58 (d, J = 9.6 Hz, 1H), 9.61 (s, 1H), 10.01 (s, 1H).

5.1.5. 7-Isopropoxy-6-methyl-4-(benzimidazole-2-ylamino)-quinoline-3-carbonitrile (8d). The compound was prepared with a 34% yield according to the method for **8a** using **6a** and 2-aminobenzimidazole. Mp 223–224 °C; EI-MS (*m*/*z*): 357.1 (M^+);^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.31–1.33 (d, J = 6 Hz, 6H), 2.30 (s, 3H), 4.97–5.01 (m, 1H), 7.15–7.20 (m, 1H), 7.34–7.36 (m, 1H), 7.58 (s, 1H), 7.63–7.69 (m, 3H), 7.84–7.87 (d, J = 8.1 Hz, 1H), 8.15 (s, 1H), 8.45 (s, 1H), 9.52 (s, 1H).

5.1.6. 7-Isopropoxy-6-methoxy-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (9a). The compound was prepared in 64% yield according to the method for **8a** using **6b** and 2-aminobenzothiazol. Mp 234 °C; EI-MS (*m*/*z*): 392.2 (M^+);^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.43–1.45 (d, J = 6 Hz, 3H), 3.99 (s, 3H), 4.72 (m, 1H), 7.40 (s, 1H), 7.63 (m, 2H), 8.01 (s, 1H), 8.12 (d, J = 8 Hz, 1H), 9.03 (d, J = 8 Hz, 1H), 9.32 (s, 1H).

Similar procedures for preparing **9b** and **9c** were carried out following the preparation methods of intermediate **9a** using **6b** and 2-amino-6-trifluoromethoxylbenzothiazole, 2-aminobenzimidazole as the reaction materials.

5.1.7. 7-Hydroxy-6-methyl-4-(pyridin-2-ylamino)-quinoline-3-carbonitrile (10). To a solution of acetic acid (20 ml) and aqueous hydrobromic acid (47%, 40 ml) heated at 120 °C was added 7 (220 mg, 0.76 mmol). Then the mixture was heated at reflux for 12 h. After cooling, the mixture was diluted with cold water (100 ml) and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column to give 115 mg product (55% yield). Mp 175–177 °C; EI-MS (*m*/*z*): 276.1 (M^+); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.33 (s, 3H), 7.25 (t, J = 7 Hz, 1H), 7.37 (s, 1H), 7.75 (d, J = 9 Hz, 1H), 7.96 (m, 1H), 8.60 (s, 1H), 9.01 (d, J = 7 Hz, 1H), 9.36 (s, 1H), 11.30 (s, 1H).

5.1.8. 7-Hydroxy-6-methyl-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (11a). To a solution of acetic acid (20 ml) and aqueous hydrobromic acid (47% yield, 40 ml) heated at 120 °C was added **8a** (300 mg, 0.80 mmol). Then the mixture was heated at reflux for 12 h. After cooling, the mixture was diluted with cold water (100 ml) and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column to give 180 mg of product (68% yield). Mp 234 °C; EI-MS (*m*/*z*): 332.1 (M^+);^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.39 (s, 3H), 7.39 (s, 1H), 7.52 (m, 2H), 7.95 (d, J = 8 Hz, 1H), 8.41 (s, 1H), 9.48 (d, J = 8 Hz, 1H), 9.56 (s, 1H), 11.50 (s, 1H).

5.1.9. 7-Hydroxy-6-methyl-4-(6-methylbenzothiazol-2-ylamino)-quinoline-3-carbonitrile (11b). The compound was prepared with a 58% yield according to the method for **11a** using **8b**. Mp 245 °C; EI-MS (*m*/*z*): 346.2 (M^+);^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.31 (s, 3H), 2.42 (s, 3H), 7.38 (m, 2H), 7.80 (s, 1H), 8.41 (s, 1H), 9.30 (d, J = 9 Hz, 1H), 9.60 (s, 1H), 9.99 (s, 1H), 11.80 (s, 1H).

5.1.10. 7-Hydroxy-6-methoxy-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (12a). A 390 mg (1.0 mmol) quantity of **9a** in 30% HBr in acetic acid (80 ml) was stirred at reflux for 12 h. After cooling, the mixture was diluted with cold water (100 ml) and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column to give 260 mg of product (75% yield). Mp 238 °C (dec); EI-MS (*m*/*z*): 348.1 (M^+); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.00 (s, 3H), 7.43 (s, 1H), 7.65 (m, 2H), 8.05 (s, 1H), 8.15 (d, J = 8 Hz, 1H), 9.05 (d, J = 8 Hz, 1H), 9.35 (s, 1H), 10.52 (s, 1H).

[Similar procedures for preparing **11c**, **11d**, **12b**, and **12c** were carried out following the preparation methods of intermediates **11a**, **11b**, and **12a** using **8c**, **8d**, **9b**, and **9c** as the stationary materials.]

5.1.11. 7-(3-Chloropropoxy)-6-methyl-4-(pyridin-2-ylamino)-quinoline-3-carbonitrile (13). A mixture of **10** (100 mg, 0.36 mmol), 1-bromo-3-chloropropane (150 μl, 1.5 mmol), and potassium carbonate (210 mg, 1.5 mmol) in DMF (10 ml) was heated at 40 °C for 3 h. The mixture was cooled, diluted with water, and extracted with ethyl acetate. The organic extracts were combined, washed with water and brine, and dried with $MgSO_4$, and the volatiles were removed by evaporation. The residue was chromatographed on silica gel with MeOH–EtOAc to give 110 mg (87% yield) of product. Mp212–214 °C (dec); EI-MS (*m*/*z*): 352.2 (M^+);^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.20 (m, 2H), 2.31 (s, 3H), 3.79 (t, J = 6 Hz, 2H), 4.24 (t, J = 6 Hz, 2H), 7.25 (t, J = 7 Hz, 1H), 7.37 (s, 1H), 7.75 (d, J = 9 Hz, 1H), 7.96 (m, 1H), 8.60 (s, 1H), 9.01 (d, J = 7 Hz, 1H), 9.36 (s, 1H), 11.30 (s, 1H).

5.1.12. 7-(3-Chloropropoxy)-6-methyl-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (14a). A mixture of **11a** (160 mg, 0.48 mmol), 1-bromo-3-chloropropane (150 μl, 1.5 mmol), and potassium carbonate (210 mg, 1.5 mmol) in DMF (20 ml) was heated at 40 °C for 3 h. The mixture was cooled, diluted with water, and extracted with ethyl acetate. The organic extracts were combined, washed with water and brine, and dried with $MgSO_4$, and the volatiles were removed by evaporation. The residue was chromatographed on silica gel with CH_3OH–EtOAc to give 135 mg (68%) of 14a. Mp 243–245 °C (dec); EI-MS (*m*/*z*): 408.2 (M^+); ^1H NMR (DMSO-d_6, 500 MHz) δ 2.20 (m, 2H), 2.39 (s, 3H), 3.79 (t, J = 6 Hz, 2H), 4.24 (t, J = 6 Hz, 2H), 7.39 (s, 1H), 7.52 (m, 2H), 7.95 (d, J = 8 Hz, 1H), 8.41 (s, 1H), 9.48 (d, J = 8 Hz, 1H), 9.56 (s, 1H), 11.50 (s, 1H).

Similar procedures for preparing **14b–d** and **15a–c** were carried out according to the preparation for intermediates **13** and **14a** using **11b–d** and **12a–c** as different starting materials.

5.1.13. 7-(3-Chloroethoxy)-6-methoxy-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (15d). A mixture of **12a** (200 mg, 0.57 mmol), 1-bromo-2-chloroethane (125 μl, 1.5 mmol), and K_2CO_3 (210 mg, 1.5 mmol) in DMF (20 ml) was heated at 40 °C for 3 h. The mixture was cooled, diluted with water, and extracted with ethyl acetate. The organic extracts were combined, washed with water and brine, and dried with $MgSO_4$, and the volatiles were removed by evaporation. The residue was chromatographed on silica gel with MeOH–EtOAc to give 176 mg (75% yield) of product. Mp 234 °C (dec); EI-MS (*m*/*z*): 410.2 (M^+); ^1H NMR (DMSO-d_6, 500 MHz) δ 3.91 (t, J = 6 Hz, 2H), 4.00 (s, 3H), 4.62 (t, J = 6 Hz, 2H), 7.43 (s, 1H), 7.65 (m, 2H), 8.05 (s, 1H), 8.15 (d, J = 8 Hz, 1H), 9.05 (d, J = 8 Hz, 1H), 9.35 (s, 1H), 10.52 (s, 1H).

5.1.14. 6-Methyl-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-(pyridin-2-ylamino)-quinoline-3-carbonitrile (16). A mixture of compound **13** (100 mg, 0.28 mmol), *N*-methylpiperazine (200 mg, 2.0 mmol), and a catalytic amount of NaI in 10 mL of DMF was heated at 80 °C for 6 h. The reaction mixture was then poured into ice water and the pH was adjusted to 8–9 by the addition of saturated $NaHCO_3$. The solids were collected by filtration to provide 75 mg of crude product. The crude product was purified by column chromatography eluting with 30% MeOH in ethyl acetate to provide 55 mg (47% yield) of compound **16** as a light yellow solid: Mp 233 °C; EI-MS (*m*/*z*): 416.1 (M^+); HRMS calcd for $C_{24}H_{28}N_6O$ 416.2325, Found 416.2334; ^1H NMR (300 MHz, D_2O): δ 2.01 (s, 3 H), 2.29–2.31 (m, 2 H), 2.95 (s, 3 H), 3.38–3.43 (br s, 4 H), 3.45–3.49 (m, 2 H), 3.54–3.64 (bs, 4 H), 3.97-4.00 (t, 2 H, J_1 = 6 Hz, J_2 = 6 Hz), 6.36 (s, 1H), 6.74–6.76 (m, 1H), 6.8–6.89 (d, 1H, J = 9 Hz), 7.23 (s, 1H), 7.64–7.66 (m, 1H), 7.75–7.78 (m, 1H), 8.15 (s, 1H); IR ν (CN) 2192 cm^{-1}.

5.1.15.6-Methyl-7-(3-morpholinopropoxy)-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (17). The compound was prepared with a 46% yield according to the method for **16** using **14a** and morpholine. Mp 210 °C; EI-MS (*m*/*z*): 460.1 $(M+1)^+$; HRMS calcd for $C_{25}H_{27}N_5O_2S$ 461.1641, Found 461.1658; ^1H NMR (300 MHz, D_2O): δ 1.76 (br s, 2H), 2.14 (s, 3H), 3.16–3.21 (m, 4H), 3.43–3.57 (m, 4H), 3.78–3.86 (m, 2H), 4.11–4.15 (m, 2H), 6.36 (s, 1H), 7.42 (m, 3H), 7.65 (m, 1H), 8.11 (s, 1H), 8.72 (s, 1H); IR ν (CN) 2187 cm^{-1}.

5.1.16. 6-Methyl-7-[3-(piperidin-1-yl)-propoxy]-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (18). The compound was prepared with a 44% yield according to the method for **16** using **14a** and piperidine. Mp 193 °C; EI-MS (*m*/*z*): 458.1 $(M+1)^+$; HRMS calcd for $C_{26}H_{29}N_5OS$ 459.1849, Found 459.1862; ^1H NMR (300 MHz, D_2O): δ 1.75 (br s, 2H), 2.02 (s, 3H), 3.15–3.20 (m, 4H), 3.43 (m, 2H), 3.52–3.56 (m, 2H), 3.78–3.87 (m, 2H), 4.11–4.15 (m, 2H), 6.34 (s, 1H), 7.40–7.45 (m, 3H), 7.64 (m, 1H), 8.10 (s, 1H), 8.71 (s, 1H); IR ν (CN) 2227 cm^{-1}.

5.1.17. 6-Methyl-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-(6-methyl-benzothiazol-2-ylamino)-quinoline-3-carbonitrile (19). The compound was prepared with a 43% yield according to the method for **16** using **14b** and *N*-methylpiperazine. Mp 272 °C; EI-MS (*m*/*z*): 487.1 $(M+1)^+$; HRMS calcd for $C_{27}H_{32}N_6OS$ 488.2114, Found 488.2148; ^1H NMR (300 MHz, D_2O): δ 1.98 (s, 3H), 2.27 (s, 5H), 3.05 (s, 3H), 3.43 (m, 2H), 3.73 (m, 8H), 3.85 (m, 2H), 6.69 (s, 1H), 7.10–7.13 (d, 1H, J = 8.2 Hz), 7.36 (s, 1H), 7.64 (s, 1H), 7.90–7.93 (d, 1H, J = 8.4 Hz), 8.93 (s, 1H); IR ν (CN) 2213 cm^{-1}.

5.1.18. 6-Methyl-7-[3-(piperidin-1-yl)-propoxy]-4-(6-methylbenzothiazol-2-ylamino)-quinoline-3-carbonitrile (20). The compound was prepared with a 48% yield according to the method for **16** using **14b** and piperidine. Mp 270 °C; EI-MS (*m*/*z*): 471.0; HRMS calcd for $C_{27}H_{29}N_5OS$ 471.2093, Found 471.2098; ^1H NMR (300 MHz, DMSO-d_6): δ 1.75 (m, 2H), 1.82 (m, 4H), 2.31 (m, 2H), 2.32 (s, 3H), 2.33 (s, 3H), 3.02 (m, 2H), 3.25–3.27 (m, 2H), 3.47–3.51 (m, 2H), 4.29–4.33 (m, 2H), 7.47–7.50 (d, 1H, J = 8.5 Hz), 7.58 (s, 1H), 7.93 (s, 1H), 8.56 (s, 1H), 8.78–8.81 (d, 1H, J = 8.5 Hz), 10.01 (s, 1H), 10.37 (br s, 1H); IR ν (CN) 2212 cm^{-1}.

5.1.19. 6-Methyl-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-[6-(trifluoromethoxy)-benzothiazol-2-ylamino]-quinoline-3-carbonitrile (21). The compound was prepared with a 64% yield according to the method for **16** using **14c** and *N*-methylpiperazine. Mp 243 °C; EI-MS (*m*/*z*): 556.2 (M^+); HRMS calcd for $C_{27}H_{27}F_3N_6O_2S$ 556.1868, Found 556.1843; ^1H NMR (300 MHz, D_2O): δ 2.13 (s, 3H), 2.39 (m, 2H), 3.05 (s, 3H), 3.50–3.51 (m, 4H), 3.57–3.59 (m, 2H), 3.63–3.69 (m, 4H), 4.10 (m, 2H), 6.51 (s, 1H), 7.38 (m, 2H), 7.43 (s, 1H), 7.45 (s, 1H), 8.22(s, 1H); IR ν (CN) 2146 cm^{-1}.

5.1.20. 6-Methyl-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-(1*H*-benzimidazole-2-ylamino)-quinoline-3-carbonitrile (22). The compound was prepared with a 61% yield according to the method for **16** using **14d** and *N*-methylpiperazine. Mp 236 °C; EI-MS (*m*/*z*): 473.1 $(M+1)^+$; HRMS calcd for $C_{26}H_{28}N_6OS$ 472.2045, Found

472.2056;^{1}H NMR (300 MHz, D_2O): δ 2.00 (s, 3H), 2.29–2.30 (m, 2H), 2.95 (s, 3H), 3.38–3.42 (m, 4H), 3.46–3.50 (m, 2H), 3.95–3.99 (m, 2H), 6.35 (s, 1H), 7.21–7.31 (m, 4H), 7.55 (s, 1H), 8.11 (s, 1H); IR v (CN) 2217 cm^{-1}.

5.1.21. 6-Methoxy-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (23). The compound was prepared with a 47% yield according to the method for **16** using **15a** and *N*-methylpiperazine. Mp 202 °C; EI-MS (*m*/*z*): 488.1 (M^+); HRMS calcd for $C_{26}H_{28}N_6O_2S$ 488.1994, Found 488.1987;^{1}H NMR (300 MHz, DMSO): δ 1.95–1.97 (br s, 2H), 2.25 (s, 3H), 2.48–2.49 (m, 10H), 3.98 (s, 3H), 4.17–4.22 (m, 2H), 7.42 (s, 1H), 7.46–7.57 (m, 2H), 7.91 (s, 1H), 7.97–7.99 (d, 1H, J = 6 Hz), 9.46–9.48 (d, 1H, J = 6 Hz), 9.52 (s, 1H), 9.98 (s, 1H); IR v (CN) 2220 cm^{-1}.

5.1.22. 6-Methoxy-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-[6-(trifluoromethoxy)-benzothiazol-2-ylamino]-quinoline-3-carbonitrile (24). The compound was prepared with a 34% yield according to the method for **16** using **15b** and *N*-methylpiperazine. Mp 262 °C; EI-MS (*m*/*z*): 572.1 (M^+); HRMS calcd for $C_{27}H_{27}F_3N_6O_3S$ 572.1817, Found 572.1835; ^{1}H NMR (300 MHz, D_2O): δ 3.03 (s, 3H), 3.60 (m, 6H), 3.67 (m, 6H), 3.88 (s, 3H), 4.18 (m, 2H), 6.91 (s, 1H), 7.40 (s, 1H), 7.49 (bs, 2H), 7.71–7.73 (d, 1H, J = 4.6 Hz), 8.28 (s, 1H), 9.05(s, 1H); IR v (CN) 2238 cm^{-1}.

5.1.23. 6-Methoxy-7-[2-(piperidin-1-yl)-ethoxyl]-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (25). The compound was prepared with a 52% yield according to the method for **16** using **15d** and piperidine. Mp 210 °C; EI-MS (*m*/*z*): 460.1 $(M+1)^+$; HRMS calcd for $C_{25}H_{27}N_5O_2S$ 461.1641, Found 461.1646; ^{1}H NMR (300 MHz, D_2O): δ 1.47–1.51 (m, 2H), 1.70–1.80 (m, 2H), 1.95–1.99 (m, 2H), 2.97–3.05 (m, 2H), 3.42 (m, 2H), 3.50–3.54 (m, 2H), 3.59 (s, 3H), 3.97 (m, 2H), 6.59 (s, 1H), 6.92 (s, 1H), 7.30–7.32 (d, 1H, J = 6.6 Hz), 7.54–7.56 (d, 1H, J = 6.9 Hz), 7.92 (s, 1H), 8.63 (s, 1H); IR v (CN) 2218 cm^{-1}.

5.1.24. 6-Methoxy-7-(2-morpholinoethoxy)-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (26). The compound was prepared with a 54% yield according to the method for **16** using **15d** and morpholine. Mp 236 °C; EI-MS (*m*/*z*): 461.0 (M^+); HRMS calcd for $C_{24}H_{23}N_5O_3S$ 461.1522, Found 461.1535;^{1}H NMR (300 MHz, D_2O): δ 1.47–1.55 (m, 2H), 1.95–2.00 (m, 2H), 2.97–3.05 (m, 2H), 3.42 (m, 2H), 3.50–3.54 (m, 2H), 3.58 (s, 3H), 3.96 (m, 2H), 6.59 (s, 1H), 6.91 (s, 1H), 7.29–7.31 (d, 1H, J = 6 Hz), 7.52–7.55 (d, 1H, J = 6 Hz), 7.92 (s, 1H), 8.62 (s, 1H); IR v (CN) 2116 cm^{-1}.

5.1.25. 6-Methoxy-7-[2-(4-methylpiperazin-1-yl)-ethoxy]-4-(benzothiazol-2-ylamino)-quinoline-3-carbonitrile (27). The compound was prepared with a 49% yield according to the method for **16** using **15a** and *N*-methylpiperazine. Mp 241 °C; EI-MS (*m*/*z*): 475.1 $(M+1)^+$; HRMS calcd for $C_{25}H_{28}N_6O_2S$ 476.1750, Found 476.1751; ^{1}H NMR (300 MHz, D_2O): δ 3.17 (s, 3H), 3.78 (m, 6H), 3.80 (m, 6H), 4.01 (s, 3H), 4.35 (m, 2H), 7.07 (s, 1H), 7.55 (s, 1H, C_5-H), 7.61–7.63 (m, 2H), 7.85–7.88 (m, 1H), 8.39–8.49 (m, 1H), (s, 1H), 9.21 (s, 1H); IR v (CN) 2086 cm^{-1}.

5.1.26. 6-Methoxy-7-[3-(4-methylpiperazin-1-yl)-propoxy]-4-(1*H*-benzimidazol-2-ylamino)-quinoline-3-carbonitrile (28). The compound was prepared with a 45% yield according to the method for **16** using **15c** and *N*-methylpiperazine. Mp 225 °C; EI-MS (*m*/*z*): 472 $(M+1)^+$; HRMS calcd for $C_{26}H_{29}N_7O_2$ 471.2383, Found 471.2378; ^{1}H NMR (300 MHz, D_2O): δ 3.01 (s, 3H), 3.43–3.46 (m, 6H), 3.58–3.65 (m, 6H), 3.85 (s, 3H), 4.15 (m, 2H), 6.87 (s, 1H), 7.37 (s, 1H), 7.47 (m, 1H), 7.69 (m, 1H), 8.24 (m, 1H), 9.02 (s, 1H); IR v (CN) 2198 cm^{-1}.

5.2. Biology

5.2.1. Src kinase assay. Src kinase activity was carried out in an ELISA format by measuring the fluorescence polarization of the reaction system (Pan Vera's Protein Tyrosine Kinase Red Assay kit, Invitrogen Corporation). Src (human recombinant, histidine-tagged 1 U/reaction; Invitrogen Corporation), reaction buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μM Na_3VO_4), poly (Glu, Tyr = 4:1) (3200 ng/mL, Sigma, St. Louis, MO, USA) substrate were added to the compound. The reaction was started by the addition of ATP (1,000 nM, Sigma) to a total volume of 20 μl and incubated at 37 °C for 30 min. Then it was stopped by addition of EDTA. Then antibody and tracer were added and the mixture was incubated at 37 °C for another 30 min. The fluorescence polarization was measured by a Magellan TECAN instrument with a Safire^2 workstation. Instructions from the manufacturer were followed for subsequent steps. Compounds were tested in triplet and the value given is an average of three determinations.

5.2.2. iNOS assay. Inducible NO synthetase activity was measured by monitoring the NO concentration generated by the induced iNOS in murine macrophages ANA-1 stimulated by exogenous LPS using a nitric oxide synthase assay kit (Beyotime Corporation). Mouse macrophages ANA-1 (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS and L-glutamine (2 mM). A total of 10^5 cells/mL were prepared the day before each experiment, and the cells were incubated at 37 °C in a humidified incubator under 5% CO_2.

Cells were then stimulated by lipopolysaccharide (20 μM; LPS, Sigma) for 24 h, and the cultured cells were collected and transferred to a 96-well plate. Assay buffer, the L-arginine substrate, and the tracer DAF-FM DA (3-amino-4-aminomethyl-2′,7′-difluorescein diacetate) were added, followed by addition of 0.1 mM NADPH to the reaction mixtures to initiate the reactions. The plate was incubated at 37 °C for 30 min, centrifuged, and washed three times in PBS. Then the fluorescence of the plate was measured by a Varioskan instrument. Instructions from the manufacturer were followed strictly, and each compound was tested in triplet; the value given is an average of three determinations.

5.2.3. SRB proliferation assays. Five tumor cell lines which were associated with overexpression of Src or iNOS were selected, including lung cancer A549, stomach cancer AGS, liver cancer HepG2, colon cancer HT-29, and prostate cancer PC-3 lines. Sulforhodamine B, trichloroacetic acid, trizma base, and acetic acid were purchased from Sigma Chemical Co. (St. Louis, USA). Lung cancer A549, stomach cancer AGS, colon cancer HT-29, and prostate cancer PC-3 cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum at 37 °C in humidified air under 5% CO_2. Liver cancer HepG2 cell line was cultured in MEM medium supplemented with 10% fetal bovine serum at 37 °C in humidified air containing 5% CO_2.

A pilot-screening operation was initiated in which the panel lines were inoculated onto a series of standard 96-well plates on day 0, in the majority of cases at 20,000 cells/well, and then pre-incubated in the absence of drug for 24 h. Test agents were then added in 10-fold dilutions starting from the highest soluble concentration, and incubated for a further 48 h. Following this, the cells were fixed in situ, washed, and dried. SRB was added, followed by further washing and drying of the stained, adherent cell mass. The inhibition of cell proliferation was assessed by measuring changes in total optical density after a culture of each cell line that was subjected to 48 h of drug treatment. The results were obtained in one independent experiment run in triplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.065.

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